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Cross-sectional study: CagA-positive *Helicobacter pylori* infection, acute coronary artery disease and systemic levels of B-type natriuretic peptide

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Abstract

Background B-type natriuretic peptide (BNP) determination is routinely used to evaluate the severity of congestive heart failure, a possible consequence of coronary artery disease (CAD). CAD originates from vascular atherosclerotic processes and is stimulated by inflammatory events, which may also be triggered by chronic bacterial infections.

Aim To explore the effect of *Helicobacter pylori* infection upon systemic BNP, tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) levels and linear homology between cardiac peptides and *H pylori*.

Methods A group of 103 consecutive patients with a diagnosis of non-ST elevation acute CAD (ACAD) and no other concomitant pathology was examined. BNP was measured by a commercial solid-phase sandwich immunoradiometric assay. *H pylori* infection, CagA serological status and circulating levels of IL-6 and TNF- α , were determined by ELISA assays. Amino acid sequence homology between human cardiac and *H pylori* peptides was investigated by Basic Local Alignment Search Tool (BLAST) analysis.

Results Circulating levels of BNP and IL-6, in pg/mL (interquartile difference), among infected patients with anti-CagA serum antibodies, respectively 781 (1899) and 37.7 (137.6), were significantly increased in respect to those measured in uninfected patients, respectively 325 (655) and 7.7 (23.5), ($p<0.01$ and $p=0.025$), and, with regard to BNP alone, also in patients infected by CagA negative *H pylori* strains, 305 (593), ($p<0.01$). TNF- α levels were raised in CagA positive in respect to uninfected patients. Tropomyosin and Ca²⁺ transporting ATPases showed strong similarities to *H pylori* proteins, suggesting the existence of molecular mimicry phenomena.

Conclusions Chronic infection by *H pylori* expressing CagA correlates with high circulating levels of BNP and IL-6 in patients with ACAD.

Introduction

Coronary artery diseases (CAD) are a major cause of disability and death worldwide. The development of markers capable of predicting the risk of CAD, such as B-type natriuretic peptide (BNP), is therefore crucial. BNP is a 32-aminoacid hormone synthesised by cardiomyocytes and the brain. Effects of BNP include natriuresis, vasodilatation, renin-angiotensin system inhibition and anti-adrenergic activity.^{1,2} Systemic BNP levels are routinely determined to evaluate the severity of congestive heart failure (CHF) and the extension and severity of acute coronary disease (CAD).^{3,4} High BNP levels indicate a reduced cardiac output and bad prognosis of CHF.^{5–7} Finally, BNP systemic concentrations may predict survival after acute myocardial infarction.^{8–10}

CAD is the final stage of an atherosclerotic process of the coronary artery walls. Atherosclerosis is a multifactorial disorder in which inflammatory diseases, including chronic bacterial infections, could play a major role.^{11,12} Infectious agents may induce atherosclerosis by increasing the levels of circulating cytokines, such as interleukin-1 (IL-1), IL-1 β receptor and its haplotypes, interleukin-6 (IL-6) and the like, leading to the stimulation of immune system-mediated responses, that is, inflammation.^{11–14}

One of the most common agents of chronic bacterial infection is *Helicobacter pylori*, a Gram-negative micro-organism present in the gastroduodenal tracts of roughly 50% of the entire world population.¹⁵ The

clinical outcome of the disease ranges from chronic gastritis to peptic ulceration and gastric cancer.^{15–17} *H pylori*-infected patients have increased systemic indices of inflammation, high neutrophil and basophil blood counts and levels of cytokines and vasoactive substances,^{18–21} which promote or aggravate the chronic inflammatory status of the gastroduodenal tract and also of organs distant from the stomach.

Epidemiological surveys have indicated that infection by *H pylori* possessing the *cagA* gene can be associated with CAD and other vascular disorders.^{22–26} *cagA* is enclosed in the so-called *cag* pathogenicity island and encodes for a highly immunogenic protein called CagA (cytotoxin associated gene A), which is endowed with carcinogenic and proinflammatory properties.^{27,28}

The pathogenetic basis of the possible association between CAD and *H pylori* infection may also reside in the existence of antigenic mimicry phenomena between bacterial peptides and some substances contained in cardiac muscle and the coronary wall.²⁹ The infection may induce antibodies and T cells to react against bacterial cell constituents, which may also react with the host's tissues, leading to immune-mediated damage. To determine the presence of antigenic cross-mimicry, we aligned bacterial epitopes with heart constituents and enzymes involved in cardiomyocyte contraction, such as troponin, calcium-transporting ATPases and so on. In cardiac tissue, as well as in the other striated muscles, the production of muscular force is chiefly controlled by changes in concentration of intracellular calcium. When calcium rises, the muscles contract, and when calcium falls, the muscles relax. Troponin, together with actin and tropomyosin, is a component of thin filaments, and is the protein complex to which calcium binds to trigger the production of muscular force. The possible presence of antibodies produced against the bacterial antigens cross-reacting with cardiac peptides involved in heart contraction, could help explaining the molecular basis of the association between *H pylori* infection and CAD.

The objective of the study was to investigate the possible relationship between *H pylori* infection, proinflammatory cytokines production and BNP levels measured in plasma samples of patients with non-ST elevation acute CAD (ACAD). We also aimed to reveal whether a structural linear homology exists between *H pylori* epitopes and cardiac proteins involved in heart contraction. If this hypothesis is fulfilled, patients with ACAD should be investigated for the *H pylori* and CagA status.

Patients and methods

Patients' characteristics

We included all eligible consecutive patients admitted during the year 2005 to our department for ischaemic heart disorders; all patients signed written informed consent to the present study.

Exclusion criteria were the presence of renal, liver and thyroid disorders and neoplastic diseases.

Heart-related exclusion causes were: history of previous myocardial infarction, recent transmural infarction and ST elevation, any isolated ST elevation and valvular diseases with haemodynamic disorder, all chronic heart failure (determined according to the New York Heart Association criteria), cardiomyopathy, systolic dysfunction with ejection fraction lower than 50%, and left ventricle (LV) hypertrophy. Finally, patients who had received antibiotics in the previous 3 months were also excluded. All patients underwent careful physical and biochemical examination, as well as electrocardiographic and echocardiographic studies (instrument Hewlett Packard, 5500 Sonos, Andover, Massachusetts, USA) by two experienced examiners, blinded to the study, to evaluate LV wall kinesis and systolic function (LV systolic function was assessed by the Simpson modified rule formula). Two to five days after admission, left cardiac catheterisation and selective coronary angiography were performed. Patients with vessel stenosis >70% of one or more coronary arteries were eligible. One hundred and three patients with non-ST elevation ACAD fulfilled the inclusion criteria; of these, 70 were men and 33 women (mean age 65 years, interval 45–75 years). Their

clinical characteristics are reported in table 1. The study was approved by the Institution's Human Scientific Committee.

Table 1 Demographic and clinical characteristics of patients examined

| | |
|--------------------------------------|----------|
| Number of patients examined | 103 |
| Men/women | 70/33 |
| Mean age (years) | 65±8 |
| Body Mass Index | 27.4±2.6 |
| Ejection Fraction | <50% |
| Plasma creatinine | 1.2±0.2 |
| Percentage of narrowed vessels | 80±8 |
| Number of patients with hypertension | 44 |
| Number of patients with dyslipidemia | 39 |
| Number of smokers | 49 |
| Number of patients with diabetes | 16 |

Biochemistry

Blood samples were collected within 24 h after admission for the most recent chest pain attack; each sample was placed in a glass tube containing 7 mL ethylene-diamine-tetraacetic acid; aprotinin was added to inhibit BNP breakdown (250 µL). All samples were stored between 3°C and 6°C, and centrifuged within 3 h at 2000 rev/min for 20 min in a refrigerated centrifuge. Platelet-poor plasma and serum were obtained and stored at -20°C. BNP was measured within 1 week after sampling by a commercial solid-phase sandwich immunoradiometric assay (Shionora BNP, Schering line), whose coefficient of variation was <10%, in the range of 3–100 pg/mL with a detection limit of 3 pg/mL of the assay. The intra-assay and inter-assay coefficients of variation were 4% and 6%, respectively. Systemic levels of IL-6 were determined by 'Human IL-6 ELISA' kit provided by Bender MedSystems, Vienna, Austria. The limit of detection was 0.92 pg/mL and the overall intra-assay and inter-assay coefficients of variation were 3.4% and 5.2%, respectively. Systemic levels of TNF-α were determined by 'Human TNF-α total' ELISA kit provided by Bender MedSystems, Vienna, Austria. The limit of detection was 2.3 pg/mL and the overall intra-assay and inter-assay coefficients of variation were 6% and 7.4%, respectively

Determination of *H pylori* infection and CagA status

The *H pylori* infectious status was determined serologically using a commercially available ELISA with a sensitivity and specificity of 96% (*H pylori* IgG, HpG screen ELISA kit, Genesis Diagnostics, Littleport, UK). Infection was confirmed by western blotting (WB) as previously described.²⁸ WB was also used to detect the presence of *H pylori* CagA antibodies in serum samples. Titres of anti-CagA antibodies were determined using 'CagA IgG ELISA Kit' provided by Genesis Diagnostics, Littleport, UK (sensitivity=96%, specificity 97%, inter-assay coefficient of variation <12%). Levels of specific antibodies were expressed by arbitrary units (AU)/mL.

Alignment of human cardiac proteins with *H pylori* proteins

We compared the linear sequence of cardiac protein with those of *H pylori* peptides to test the hypothesis that molecular mimicry mechanisms may account, in part, for the supposed cardiac tissue damage in people infected by this bacterium. The cardiac proteins we chose to be aligned with *H pylori* peptides are constituents of the heart tissue or functional proteins that enable the organ to contract. Should some of them share linear homology with bacterial peptides, infected patients could produce cross-reactive antibodies during the infection, which could injure the heart tissue and damage the function of certain enzymes, thus contributing to determine the development of heart failure.

In order to find regions of similarity between amino acid sequences, we used the BLAST (Basic Local Alignment Search Tool) programme in the protein databases at the National Center for Biotechnology Information (NCBI; Bethesda, Maryland, USA; <http://www.ncbi.nlm.nih.gov>).³⁰ The programme compares protein sequences to sequence databases and calculates the statistical significance of matches. BLAST results can also be used to infer functional and evolutionary relationships between sequences.³⁰

Sequences longer than five aminoacids have also been included, even if the alignment was interrupted by one or two non-matching aminoacids. The programme also takes into consideration different, though antigenically equivalent ('similar'), aminoacids. Results include percentages of identical and similar aminoacids and the number of intervals lacking linear homology between two homologue sequences ('gaps'). The higher the percentage of identical or similar aminoacids and the lower the number of gaps, the greater is the structural homology between two proteins.

The aligned cardiac proteins are the following.

Amino acid sequences of the following human cardiac proteins were aligned with *H pylori* proteins.

- chain A, Solution nuclear magnetic resonance (NMR) Structure Of The C-Terminal-Hand Domain of Human Cardiac Sodium Channel Nav1.5
- cardiac calsequestrin 2 precursor [*Homo sapiens*]
- troponin I, cardiac [*H sapiens*]
- troponin T, cardiac [*H sapiens*]
- troponin T type 2, cardiac isoform 1 [*H sapiens*]
- cardiac muscle ryanodine receptor [*H sapiens*]
- Actin, α cardiac muscle 1; AltName: Full= α -cardiac actin
- actinin, α 1 isoform b [*H sapiens*]
- S100 calcium-binding protein A6 [*H sapiens*]
- ATPase, Ca^{2+} transporting, slow twitch 2 isoform 2 [*H sapiens*]
- calcium channel, voltage-dependent, L type, α 1C subunit isoform 13 [*H sapiens*]
- myoglobin [*H sapiens*]
- potassium channel, subfamily K, member 3 [*H sapiens*]
- myotrophin [*H sapiens*]
- Chain B, C-Domain Of Human Cardiac Troponin C In Complex With The Inhibitory Region Of Human Cardiac Troponin I
- Chain A, C-Domain Of Human Cardiac Troponin C In Complex With The Inhibitory Region Of Human Cardiac Troponin I
- Chain I, Structure Of The Regulatory N-Domain Of Human Cardiac Troponin C In Complex With Human Cardiac Troponin-I(147–163) And Bepridil
- Chain A, Solution NMR Structure Of The C-Terminal EF-Hand Domain Of Human Cardiac Sodium Channel Nav1.5
- Chain C, NMR Solution Of The Regulatory Domain Cardiac F77w-Troponin C In Complex With The Cardiac Troponin I 144–163 Switch Peptide
- Chain A, Crystal Structure Of Recombinant Human Placental Annexin V Complexed With K-201 As A Calcium Channel Activity Inhibitor
- ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1 [*H sapiens*]
- Similar to ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1 [*H sapiens*]

***H pylori* strains whose peptides were aligned with human cardiac proteins**

- *Helicobacter pylori* J99 (taxid:85963)
- *Helicobacter pylori* 26695 (taxid:85962)
- *Helicobacter pylori* HPAG1 (taxid:357544)

- *Helicobacter pylori* SS1 (taxid:102617)

Statistical analysis

The data are reported as median and interquartile differences (75°–25° percentile). The normality of distribution was tested by the Shapiro–Wilk test. Comparisons concerning levels of circulating BNP, IL-6, and TNF- α between *H pylori* infected and uninfected patients, or between CagA positive (CagA+) and CagA negative (CagA-) patients, were performed by the Mann–Whitney U test. Correlations between levels of antibodies to overall *H pylori* antigens and the CagA protein were estimated by the Spearman r coefficient. A p value<0.05 (two-tailed) was considered statistically significant. All analyses were elaborated by SPSS statistical software V.13 (SPSS, Chicago, Illinois, USA).

Statement of responsibility

Authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

A total of 103 patients fulfilled the enrolment criteria. Demographic and clinical characteristics of patients are reported in table 1.

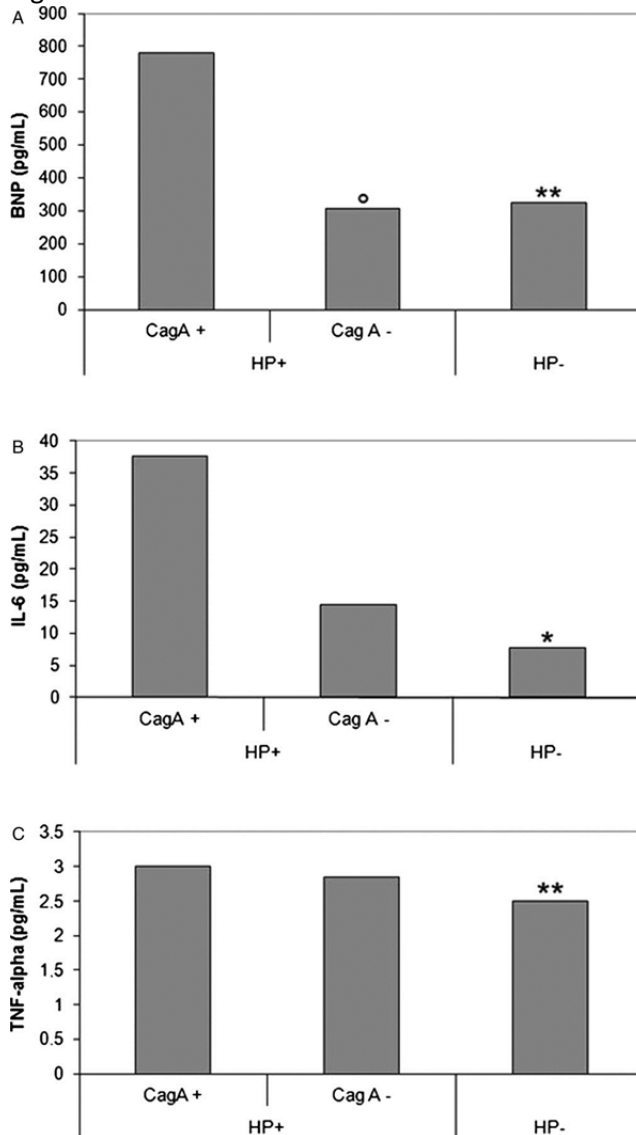
Serodiagnosis of *H pylori* infection and CagA status

A total of 41 patients (39.8%) were *H pylori* positive, and 19 of these patients (46.3%) had serum antibody to CagA (CagA+); 22 infected patients (53.6%) tested negative for anti-CagA antibodies (CagA-). Overall, 62 patients (60.1%) were uninfected.

Levels of circulating BNP, IL-6 and TNF- α

BNP median levels, in pg/mL (in parentheses, the interquartile difference), in *H pylori* infected and uninfected patients were 601 (678) and 325 (655), respectively ($p=0.178$); median levels in infected CagA+ and CagA negative (CagA-) patients were 781 (1899) and 305 (593) ($p<0.01$). The difference between BNP levels in infected CagA+ patients and uninfected patients was also statistically significant ($p<0.01$, figure 1A).

Figure 1



Median distributions of B-type natriuretic peptide (BNP) (A), IL-6 (B) and TNF- α (C) levels in *Helicobacter pylori* infected and uninfected patients and in CagA+ and CagA- infected patients. ° $p < 0.01$ CagA+ versus CagA-; * $p < 0.05$; CagA+ versus HP-; ** $p < 0.01$ CagA+ versus HP-. HP, *Helicobacter pylori*.

IL-6 median levels (in pg/mL) among infected and uninfected patients were 17.0 (71.7) and 7.7 (23.5) ($p = 0.025$), respectively; median levels in infected CagA+ and CagA- patients were 37.7 (137.6) and 14.5 (16.0), respectively ($p = 0.320$, non-significant). The difference between IL-6 median levels in infected CagA+ and uninfected patients was significant at p level < 0.05 (figure 1B).

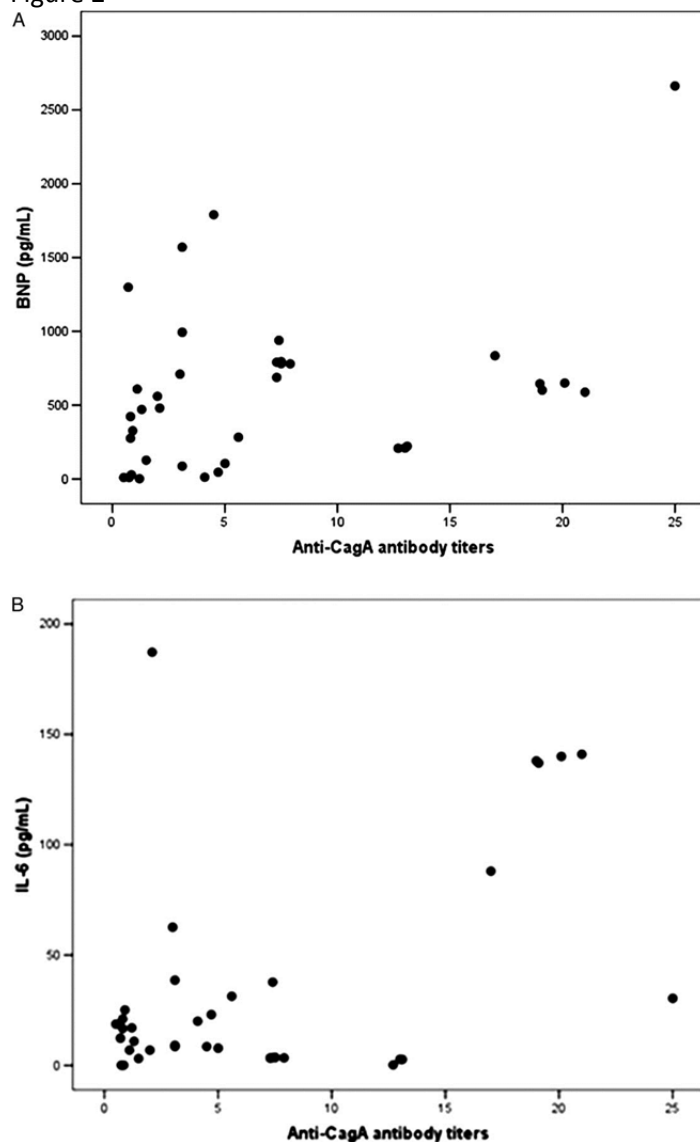
TNF- α median levels in infected and uninfected patients were 2.9 (1.9) and 2.5 (1.3), respectively ($p < 0.01$). Levels in infected CagA+ and CagA- patients were 3.0 (3.1) and 2.8 (1.6), respectively ($p = 0.244$). The difference between TNF- α mean levels in infected CagA+ and uninfected patients was significant ($p < 0.01$, figure 1C).

Correlations between BNP and IL-6 with levels of antibodies to overall *H. pylori* antigens and CagA protein

Concentrations of BNP and IL-6 in the bloodstream correlated significantly with the levels of antibodies to overall *H. pylori* antigens ($r = 0.258$, $p < 0.05$ and $r = 0.279$, $p < 0.05$, respectively) and even more strongly with

the levels of antibodies to the CagA protein ($r=0.802$, $p<0.001$ and $r=0.620$, $p<0.001$) (figure 2A,B; some observations were not reported in figures because they were outliers). Correlations between levels of TNF- α and anti-*H. pylori* overall antigens and anti-CagA did not reach statistical significance ($r=-0.052$, $p=0.675$ and $r=-0.086$, $p=0.702$).

Figure 2



Scatterplots of the relationship between B-type natriuretic peptide (BNP) (A) and IL-6 (B) levels, and levels of antibodies against CagA. (A) $r=0.802$, $p<0.001$; (B) $r=0.620$, $p<0.001$.

Amino acid alignments

To compare the aminoacid sequences we used the BLAST programme. By this method, the aminoacid linear sequences of each cardiac protein is compared with those of *H. pylori* peptides. When two proteins share five (or more) identical or chemically similar aminoacids in a row, the programme considers these sequences homologue and reports their position in the protein.

Many cardiac peptides shared partial structural homologies with *H. pylori* proteins.

Three human cardiac polypeptides showed a highly significant linear homology with peptides expressed by *H pylori* J99 (the bacterial gene encoding the proteins that aligned with the cardiac proteins is reported in parentheses):

- ATPase, Ca²⁺ transporting, cardiac muscle, fast twitch 1 (NP_223445.1)
- similar to ATPase, Ca²⁺ transporting, cardiac muscle, fast twitch 1 (NP_223072.1)
- tropomyosin=33 kDa calcium-binding protein fragment (NP_223965.1).

Bacterial polypeptides that showed a significantly linear homology ($E < 0.05$) with the above reported cardiac proteins are reported in table 2. Alignments with the other *H pylori* strains gave similar results.

Table 2 Human cardiac polypeptides displaying the most significant homologies with *Helicobacter pylori* J99 proteins

| Sequences producing significant alignments | Bits* E value |
|---|---------------|
| ATPase, Ca ²⁺ transporting, cardiac muscle, fast twitch 1 | |
| Putative heavy-metal cation-transporting P-type ATPase | 46.2 2e-06 |
| Copper-transporting P-type ATPase | 39.7 2e-04 |
| Similar to ATPase, Ca ²⁺ transporting, cardiac muscle, fast twitch 1 | |
| Copper-transporting P-type ATPase | 57.8 1e-09 |
| Putative heavy-metal cation-transporting P-type ATPase | 53.5 2e-08 |
| Putative component of cation transport for cbb3-type oxidase | 41.6 9e-05 |
| Tropomyosin=33 kDa calcium binding protein fragment | |
| Hypothetical protein jhp1247 | 26.9 0.037 |

*Bits=Bit-score: A log-scaled version of a score. The score is a number used to assess the biological relevance of a finding. In the context of sequence alignments, a score is a numerical value that describes the overall quality of an alignment. Higher numbers correspond to higher similarity.

Discussion

The serum BNP level is one of the most widely used blood markers whenever a severe heart dysfunction is suspected; it is routinely employed by cardiology specialists, by internal medicine physicians, and general practitioners. The validity of BNP determination in CAD is provided by its extensive use in medicine.^{1–10}

The main result of the present study is that patients with non-ST elevation ACAD, infected by *H pylori* strains expressing CagA, have the highest BNP systemic levels. This finding is novel and may have important consequences for this subgroup of patients. Should it be confirmed by other studies, it could pave the way for a new therapeutic intervention, that is, the cure of *H pylori* infection for patients presenting an increased risk of unfavourable coronary events. Another interesting finding is that IL-6 and BNP concentrations strictly correlate with the titres of anti-CagA antibodies. Such observations suggest that, in case of cardiac patients infected by CagA expressing organisms, those with the strongest immune response to CagA may be exposed to the most serious outcomes of the heart disease.

IL-6 high levels observed in infected patients seropositive for CagA deserve further comment. IL-6 has a crucial role in the development of atherosclerosis and ischaemic heart disorders: it is directly capable of inducing BNP mRNA and protein secretion by cultured rat cardiomyocytes³¹; levels of IL-6 contribute to short-term and long-term mortality of acute and chronic heart failure,^{31,32} and circulating levels of IL-6 can predict future myocardial infarction.^{33–35} Ischaemic heart disorders are the consequence of an atherosclerotic process. A concomitant cause of atherosclerosis is inflammation. Infections represent the single most frequent determinant of inflammation. In case of *H pylori* infection, the organism colonises the

human stomach for life (if infection is not properly treated); therefore, the trigger is continuous and inflammation lasts for a lifetime. These considerations may help to understand the importance of *H pylori* infection in atherosclerotic heart disease.

Tests for amino acid linear homology showed a strong similarity between *H pylori* peptides and human tropomyosin chain and cardiac ATPases involved in calcium transport, highlighting the possibility of molecular mimicry phenomena that may end damaging the cardiomyocytes. More specifically, the homology with tropomyosin resides in the protein domain capable of binding calcium. Calcium mobilisation is mandatory for all muscle contraction-release and is of crucial importance in cardiomyocytes, given the additional need for a long plateau of calcium-induced potential. This is not the first example of antitropomyosin antibodies developed during autoimmune or inflammatory diseases. Autoantibodies against tropomyosin may also occur among patients with autoimmune hepatitis and patients with ulcerative colitis, in which the immune cross-reaction was shown to be of paramount importance in the pathogenesis of colonocyte death.^{36–38} The presence of antibodies potentially cross-reacting with cardiac ATPases regulating the calcium turnover could disorganise the heart contraction and cause damage, because calcium handling is mandatory for the function of any muscle.³⁹

Even if BNP is not very specific, it is produced for the most part by cardiomyocytes (and in small part by the brain)^{1,2}; thus, high levels of this peptide in patients with cardiac disorders and no other concomitant pathology can be considered a marker of reduced cardiac output, and its concentrations may predict survival after acute myocardial infarction.^{3–10} In this sense, it can be considered specific. We are aware that further studies are needed to confirm these findings; however, our speculations are reinforced by numerous reports of heart diseases caused by antibodies against heart antigens: (a) severe heart failure is associated with reduced mRNA levels and protein expression of sarcoplasmic reticulum Ca^{2+} ATPase in an animal model;⁴⁰ (b) maternal antibodies against cardiac antigens may cause the embryonic heart to stop beating^{41,42}; (c) autoantibodies to heart proteins have an important role in the development of dilative cardiomyopathy (DCM): 40% of patients with DCM and 17% of those with ischaemic heart disease had a strong antiheart IgG response to myosin light chain, tropomyosin, actin, HSP-60, unidentified protein and myosin heavy chain.⁴³ These examples show that the possible damage for heart deriving by cross-reactive antibodies is not hypothetical, but is real.

In conclusion, Italian patients with non-ST elevation ACAD have much higher circulatory levels of BNP and IL-6 if they are infected by *H pylori* strains expressing CagA. In addition to the secretion of high systemic concentrations of IL-6, the occurrence of molecular mimicry phenomena could help explaining the high BNP levels detected in infected patients. Since the worst left ventricular performance corresponds to the highest BNP level, we hypothesise that infected patients with serum antibodies to CagA will progress more rapidly to LV malfunction. As a corollary, we suggest that diagnosis and cure of *H pylori* infection should be incorporated into the work-up of the 'at risk' patients prior to myocardial infarction or other severe outcomes of coronary disorders.

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